

EXHIBIT "A"

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In This Issue

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Life and Death on the Epidermal Edge: New Transgenic Mouse Model Resembling Graft Versus Host Disease (GVHD) Confirms Importance of Location, Location, Location

Since skin covers the body, it serves as a primary physical barrier protecting us against physical, chemical, and infectious assaults. In addition, skin contains an abundance of immunocytes located both within the epidermis and dermis to provide both innate and adaptive immune responses should a deleterious agent breach the stratum corneum, and enter vital tissue sites in skin. The presence of immunocytes including dendritic antigen presenting cells that are highly motile in both epidermis (Langerhans cells (LC)) and dermis (dermal dendritic cells), together with resident memory T cells (naïve T cells are largely excluded from peripheral tissue sites), presents a dilemma. On the one hand, these professional immunocytes are well positioned to handle foreign invaders, but these same cells may also trigger and mediate autoimmune reactions to self-derived peptides or glycolipids generated during the course of an inflammatory skin response. Given this dilemma, it should not be surprising that the skin is one of the most frequent organ sites beset by persistent T cell-mediated chronic inflammatory diseases thought to reflect autoimmune reactions. The precise molecular basis for this breakdown in immunological tolerance is an area of active research by Immunodermatologists.

In this issue, a team of immunodermatologists led by Dr Stephen Katz (p 109) characterizes a transgenic mouse model in which the epidermal keratinocytes are genetically engineered to express chicken ovalbumin in the basal layer. When T cells from another transgenic mouse strain that are engineered to express a T cell receptor uniquely capable of recognizing ovalbumin are injected intravenously, these injected T cells move from the circulation and infiltrate the skin and esophagus. Inflammatory reactions occur at these sites, but not in other tissues such as liver, lung, intestine, etc. At least two other important observations were made in this study. First, the cutaneous inflammation occurred without requiring any pre-conditioning of skin following intravenous administration of the T cell clone; and secondly, if the T cells were injected intradermally, no significant inflammatory reaction ensued.

The cutaneous reaction occurring in this model resembled GVHD, as the intravenously administered CD8+ T cells migrated into the skin and triggered a premature apoptotic response of epidermal keratinocytes. As such, this new transgenic model may not only contribute to our

understanding of tolerance induction in skin, it may also shed light on the immunopathogenesis of acute cutaneous GVHD. GVHD is well worth studying, as it is a major cause of morbidity and mortality in allogeneic bone marrow transplantation (alloBMT). Mechanisms that decrease or eliminate GVHD while preserving graft versus tumor effects and enhancement of immune reconstitution would be a welcome addition for improving the safety and efficacy of alloBMT.

Returning to the observations made by Katz *et al*, the failure of direct intradermally injected CD8+ ovalbumin-responsive T cells to trigger an immune response, put together with the fact that identical CD8+ T cells intravenously injected did provoke a prominent cutaneous inflammatory reaction, is intriguing. The authors suggest the basis for this highly divergent response is due to the importance of epidermal LC that are required to carry the keratinocyte-derived antigen (ovalbumin) to the regional lymph node. Presumably the microenvironment in this extracutaneous location is conducive to activation of the circulating CD8+ T cells, whereas in non-inflamed skin, these migrating T cells are not locally activated. Given the availability of other transgenic mice with reduced or absent epidermal LC, this hypothesis could be tested. It would also be interesting to determine if pre-conditioning the mouse skin with the kinds of preparative regimens used to ablate recipients' bone marrow prior to transplantation, such as high-intensity γ irradiation, or chemotherapeutic administration, would create any danger signals in the skin, and thereby facilitate local activation of intradermally injected CD8+ T cells.

In any event, there are several informative lessons to be gleaned from this report, and as any real estate agent or land developer will tell you, location, location, location often determines whether a transaction will be successful or not. From this report, it appears that the location in which the peptide antigen derived from ovalbumin is presented will determine if a cutaneous inflammatory reaction will develop.

Signaling Pathways Regulating Cutaneous Carcinogenesis Reveal Important Roles for Survival Factors and the Cytokine TNF- α

Epithelial-derived cancers arising in skin represent one of the most common neoplasms in humans. Thus, considerable effort by a wide range of investigators has focused on defining molecular events that contribute to the emergence of malignant cells derived from epidermal keratinocytes. Two important lines of inquiry involve: (1) characterizing the response of keratinocytes to ultraviolet irradiation because

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of the strong epidemiological links between sun exposure and development of skin cancer and (2) determining the role of chronic inflammation in the pathogenesis of skin cancer, because sustained inflammation has been connected to cancer development.

In this issue, there are two interesting reports that deal with each of these topics. Decraene *et al* (p 207) examine the apoptotic response of two different squamous carcinoma cell lines that carry p53 mutations, and compare these responses with normal primary human keratinocytes. Since these investigators, and others, have observed a role for the survival pathway mediated by AKT activation, they initially interrogate these cell lines focusing on AKT. In one cell line, A431, the malignant cells could not phosphorylate a key amino acid (i.e., T308), and hence the cells were highly sensitive to apoptotic induction following UV-light exposure. But the other malignant cell line A253 constitutively phosphorylated this residue, and these cells were relatively resistant to UV-light induced apoptosis. Even when the A431 cells were stimulated with a mitogen (Insulin-like growth factor-1), they could not phosphorylate T308, and hence failed to acquire a death-defying phenotype. As previous reports indicated that wild-type p53 could negatively regulate AKT activity, the stable re-expression of wild-type p53 was examined in both cell lines, but none of the aforementioned responses appeared to require functional p53 (although the mutant p53 present in the T cells may act as a dominant negative for wild-type p53). Since one of the prevailing dogmas in cutaneous oncology involves a central role for p53 as a tumor suppressor, reducing cancer formation by promoting apoptosis of keratinocytes bearing UV-light induced DNA mutations, the current results suggest it may be appropriate to re-examine the precise molecular role for p53 in cutaneous carcinogenesis. Also, these results suggest therapeutic agents targeting the AKT kinase pathway may be capable of triggering apoptotic reactions in malignant cells, although it will be important not to perturb the homeostatic planned cell death pathway in adjacent normal keratinocytes.

In the report by van Hogerlinden *et al* (p 101) characterization of a transgenic mouse strain is performed in which the basal layer keratinocytes are genetically engineered such that the NF- κ B signaling pathway is inhibited, compared with littermate control mice, all of the transgenic mice with impaired NF- κ B activation developed invasive tumors accompanied by chronic inflammatory cell infiltration in the skin. While the loss of NF- κ B signaling would have been predicted to produce enhanced keratinocyte proliferation, the associated enhanced levels of the cytokine TNF- α was a bit surprising, since cytokine production is generally dependent on NF- κ B signaling. It was also surprising that these transgenic mice routinely develop skin cancers without requiring exposure to UV-light or chemical irritants to trigger Ras activation or treatment with tumor promoters. Given the concomitant presence of chronic inflammation, the authors speculate on the role of inflammation in the transformation process, particularly highlighting a role for the cytokine TNF- α . Besides this report, another report also employing transgenic mice with abnormal NF- κ B signaling (Pasparakis *et al*, *Nature* 417:861-866, 2002) observed a chronic inflammatory skin

phenotype (resembling psoriasis), which was dependent on TNF- α , as crossing these mice with a transgenic strain lacking TNF receptor I resulted in complete prevention of the phenotype.

I raise this other report for two reasons. First, the current authors also indicate in unpublished results that prevention of skin cancer occurs when their mice are crossed to mice lacking TNF-receptor I; and second, because psoriasis appears to represent a TNF- α -dependent chronic inflammatory skin disease, but psoriatic plaques generally fail to develop into skin cancers. Thus, there appears to be a "fly in the ointment" as investigators attempt to link chronic inflammation to development of skin cancer. Indeed, the current report in this issue points out that the flaky skin mutant mouse, and other transgenic mice with chronic skin inflammation, do not develop skin cancer. Clearly, more work is required to determine if TNF- α -dependent inflammation is necessary, but not sufficient, for skin cancer development in either mice or men and if a more precise role for TNF- α in the inflammatory response that is linked to cancer exists.

Furthermore, with the growing use of a new class of biological-based therapies which target TNF- α in patients with psoriatic arthritis, rheumatoid arthritis, inflammatory bowel disease, and psoriatic skin disease, it should become obvious fairly quickly if suppressing TNF- α is not only good for soothing chronic inflammation, but also for reducing or preventing the development of skin cancer. Initially, many of us were concerned that blocking TNF- α would reduce immunosurveillance, and as is seen in immunocompromised transplant patients, there would be increased, rather than decreased, number of skin cancers. But in light of the aforementioned results in mice (and only rare clinical reports in patients) the jury is still out on the precise role for cutaneous TNF- α -mediated inflammation and skin cancer. Only clinical vigilance and further experimentation will provide us with more definitive answers to this critically important issue that not only impacts chronic inflammation, but also cancer development and treatment.

Tape Stripping of Human Skin with a Molecular Twist

In the past, dermatologists and investigative skin biologists made use of repeated tape stripping of human skin to study the Koebner phenomenon in patients with psoriasis, and to study the effect of barrier perturbation on cytokine cascades initiated by epidermal keratinocytes. Regarding the pathogenesis of psoriasis, it was observed that in a subset of patients, if the symptomless or pre-psoriatic skin was repeatedly tape stripped to remove the most superficial layer of epidermis (i.e., the stratum corneum responsible for maintaining the barrier function of skin), then a psoriatic lesion would be created. Unfortunately, such induction of psoriatic lesions was not always predictable, which limited its usefulness as an investigative tool. Nonetheless, the fact that such minor trauma to the symptomless could trigger the onset of psoriatic lesions in some patients permitted delineation of early cellular events, which was helpful to immunodermatologists in the pioneering days of psoriasis clinical research.

The second use of cellophane tape was to determine if removal of the stratum corneum was necessary and sufficient to trigger cytokine release by epidermal keratinocytes. These studies revealed in both human and mouse skin that tape stripping followed by punch biopsies processed for RT-PCR, or immunostaining, triggered keratinocytes to rapidly produce primary cytokines such as TNF- α . Prior to these results, it was widely believed that keratinocytes were immunologically inert (i.e., sub-served a brick and mortar function), and only represented targets for inflammatory cytokines, and not instigators of inflammatory reactions. But the clear-cut demonstration using the tape stripping method *in vivo* refuted this notion, and opened the way for additional studies demonstrating unequivocally that keratinocytes possessed an impressive array of immunological attributes including cytokine production, adhesion molecule expression and co-stimulatory function—even presenting bacterial-derived superantigens to T lymphocytes.

In this Issue, Wong *et al* return to the use of adhesive tape by devising a substitute for invasive punch biopsies to retrieve RNA from skin cells in a non-invasive fashion. While Vera Morhenn had previously established that tape stripping 20 times could lead to the recovery of sufficient RNA for mRNA analysis, she and her colleagues in the current report (p 159) extend this line of inquiry by only using four sequential applications of tape to human skin. They documented the utility of this elegant approach by performing a small clinical trial in which skin was pre-treated with either 1% sodium lauryl sulfate (SLS) or water (control) under occlusion. Furthermore, they compared and contrasted the molecular results obtained after four sequential tape strippings to results obtained following shave biopsies.

While tape stripping probably reflects molecular events in the superficial epidermal layers and hair follicles, shave biopsies sample a deeper and more heterogeneous cell population. Nonetheless, both non-invasive and invasive sampling procedures yielded enhanced RNA levels for IL-1 β and IL-8 in SLS-mediated irritated skin; although the relative fold changes varied widely.

Perhaps of greater significance, the investigator determined that low RNA yields from the non-invasive tape stripping sampling method could be combined with DNA microarray analysis to produce gene expression profiles for normal and irritated skin. Such a comprehensive approach may pave the way for follow-up studies comparing and contrasting the molecular profile of irritant contact *versus* allergic contact dermatitis responses in human skin. Indeed, as the practice of molecular medicine evolves, such non-invasive skin sampling coupled with customized arrays may permit diagnostic and prognostic evaluations to be performed for a wide variety of benign, inflammatory, and malignant skin disorders.

While it may be premature to discard our punch biopsies or light microscopes and install microarray chips in our clinics and pathology laboratories, a day may come when we look into our microscopes and not only see cellular arrays, but also molecular profiles. Whether we look for these molecular patterns, or whether we see molecular patterns may depend on our perspectives and imaginations, rather than limitations in the use of this amazing technological advancement.

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